Characteristics of GABAergic and cholinergic neurons in perinuclear zone of mouse supraoptic nucleus

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Wang L, Ennis M, Szabó G, Armstrong WE. Characteristics of GABAergic and cholinergic neurons in perinuclear zone of mouse supraoptic nucleus. J Neurophysiol 113: 754–767, 2015. First published November 5, 2014; doi:10.1152/jn.00561.2014.—The perinuclear zone (PNZ) of the supraoptic nucleus (SON) contains some GABAergic and cholinergic neurons thought to innervate the SON proper. In mice expressing enhanced green fluorescent protein (eGFP) in association with glutamate decarboxylase (GAD)65 we found an abundance of GAD65-eGFP neurons in the PNZ, whereas in mice expressing GAD67-eGFP, there were few labeled PNZ neurons. In mice expressing choline acetyltransferase (ChAT)-eGFP, large, brightly fluorescent and small, dimly fluorescent ChAT-eGFP neurons were present in the PNZ. The small ChAT-eGFP and GAD65-eGFP neurons exhibited a low-threshold depolarizing potential consistent with a low-threshold spike, with little transient outward rectification. Large ChAT-eGFP neurons exhibited strong transient outward rectification and a large hyperpolarizing spike afterpotential, very similar to that of magnocellular vasopressin and oxytocin neurons. Thus the large soma and transient outward rectification of large ChAT-eGFP neurons suggest that these neurons would be difficult to distinguish from magnocellular SON neurons in dissociated preparations by these criteria. Large, but not small, ChAT-eGFP neurons were immunostained with ChAT antibody (AB144P). Reconstructed neurons revealed a few processes encroaching near and passing through the SON from all types but no clear evidence of a terminal axon arbor. Large ChAT-eGFP neurons were usually oriented vertically and had four or five dendrites with multiple branches and an axon with many collateral and local arborizations. Small ChAT-eGFP neurons had a more restricted dendritic tree compared with parvocellular GAD65 neurons, the latter of which had long thin processes oriented mediolaterally. Thus many of the characteristics found previously in unidentified, small PNZ neurons are also found in identified GABAergic neurons and in a population of smaller ChAT-eGFP neurons.

interneurons; GABA; acetylcholine; oxytocin; vasopressin

Unlike many areas of the central nervous system, the rodent supraoptic nucleus (SON) of the hypothalamus does not possess a well-characterized population of interneurons. Most SON neurons are considered magnocellular neurosecretory cells (MNCs; ≥20-μm soma diameter), synthesize oxytocin (OT) or vasopressin (VP), possess one to three sparsely branching dendrites, and project an axon to the neurohypophysis, where these hormones are released near fenestrated capillaries for systemic distribution (Armstrong 1995, 2014). In Golgi studies of rat, Bruni and Perumal (1984) and Dyball and Kempley (1982) described a few smaller neurons in the SON with a different morphology, and similar neurons have been observed in rabbit (Felten and Casher 1979) and monkey (LuQui and Fox 1976). Iijima and Saito (1983) also described a small group of neurons that stained histochemically for GABA transaminase, unlike the MNCs.

In contrast to the paucity of evidence for classic interneurons within the SON, investigators have suggested that the perinuclear zone (PNZ) immediately dorsal to the SON contains neurons that project to the SON and could functionally serve as interneurons. Small tracer injections into the SON retrogradely label PNZ neurons (Iijima and Ogawa 1981; Jhamandas et al. 1989; Raby and Renaud 1989; Tribollet et al. 1985), and transneuronal transport of pseudorabies virus following neurohypophysial injections has been observed in the PNZ (Levine et al. 1994). Complementarily, anterograde transport of the plant lectin Phaseolus vulgaris leucoagglutinin from the PNZ to the SON has been reported (Roland and Sawchenko 1993). Neurons in this region could account for the large number of intact synapses remaining in the SON after its surgical isolation (Lérant et al. 1975). The PNZ contains GABAergic neurons (Tappaz et al. 1983; Theodosis et al. 1986) that are thought to mediate the rapid inhibition of VP neurons following transient hypertension (Jhamandas et al. 1989; Nissen et al. 1993). Although anatomical evidence is lacking, glutamatergic PNZ neurons also have been postulated, since local stimulation of these regions can produce inhibitory or excitatory postsynaptic potentials in SON neurons (Boudaba et al. 1997; Wuarin 1997). Finally, a group of cholinergic neurons was identified in the PNZ with processes projecting into the SON (Mason et al. 1983). While these were later described as dendrites rather than synapse-forming axons (Meeker et al. 1988; Theodosis and Mason 1988), stimulation of the PNZ does evoke monosynaptic excitatory synaptic potentials in the SON blocked by selective nicotinic receptor antagonists, and inhibition of acetylcholinesterase activity increases excitatory activity in the SON, even when glutamate receptors are blocked (Hatton and Yang 2002). These actions, as well as direct actions of nicotine (Zaninetti et al. 2002), are mediated by α7 nicotinic receptors on both OT and VP neurons and likely underlie the actions attributed to nicotinic activation of VP release (Sladek and Joynt 1979a, 1979b).

We previously characterized rat PNZ neurons with small somata and very diverse dendritic morphologies, using intracellular recording and biocytin labeling in hypothalamo-neurohypophysial explants. Despite this diversity, a commonality in their electrophysiological properties was the relative lack of fast outward rectification coupled with the presence of low-
threshold depolarizations (Armstrong and Stern 1997). In the present study we used three strains of transgenic mice to study PNZ neurons containing synthetic enzymes for GABA [glutamate decarboxylase (GAD)65 or GAD67] or for acetylcholine [choline acetyltransferase (ChAT)], the promoters of which were tagged with the fluorescent marker enhanced green fluorescent protein (eGFP). We then recorded from identified GAD or ChAT neurons to compare their electrophysiological characteristics with one another and with unidentified PNZ neurons previously described (Armstrong and Stern 1997).

**MATERIALS AND METHODS**

**GAD65-eGFP-Expressing Transgenic Mice**

Transgenic mice expressing GAD65-eGFP were maintained as a breeding colony by M. Ennis at the University of Tennessee Health Science Center (UTHSC) and were originally provided by G. Szabó. A description of these mice can be found in López-Bendito et al. (2004), and numerous articles have been published on brain GABAergic anatomy and function using this line (e.g., Bali et al. 2005; Betley et al. 2009; Cui et al. 2011; Parrish-Aungst et al. 2007; Shim et al. 2007, 2011; Wierenga et al. 2010; Zhang et al. 2006). The Szabó lab generated several lines of GAD65 mice—those used here were from line 30 and have been found to substantially overlap in hypothalamus and elsewhere with the known distribution of neurons immunoreactive for GAD or GABA (e.g., Mugnaini and Oertel 1985).

**GAD67-eGFP-Expressing Transgenic Mice**

Transgenic mice expressing GAD67-eGFP were purchased from Jackson Lab (Bar Harbor, ME; strain CB6-Tg(Gad1-EGFP)G42Zjh/J) and are described in detail on the Jackson Lab website (http://jaxmice.jax.org/strain/007677.html). They were maintained at UTHSC in a colony by Dr. Fuming Zhou. Like the GAD65 mice, this transgenic line has been used previously (e.g., Ango et al. 2004; Brenneman and Maness 2008; Starostik et al. 2010).

**ChAT-eGFP-Expressing Transgenic Mice**

Transgenic mice expressing ChAT(BAC)-eGFP were also purchased from Jackson Lab [strain: B6.Cg-Tg(RP23–268L19-EGFP)2Mik/J] and were maintained in a colony at UTHSC by Drs. Fuming Zhou and Kazuko Sakata. Details for their development can be found on the Jackson Lab website (http://jaxmice.jax.org/strain/010802.html). These mice have been used previously to characterize cholinergic neurons (e.g., Ade et al. 2011; Bacskai et al. 2014; Nagy and Aubert 2012, 2013; Tallini et al. 2006).

**Animal Care**

All animals housed at the UTHSC facility must be received pathogen free, and sentinels are routinely tested in these quarters to maintain a pathogen-free environment. Animals were group housed (4 or 5 per cage) and given free access to water and food. The Institutional Animal Care and Use Committee (IACUC) at UTHSC approved the protocols in this study.

**Slice Preparation**

Coronal slices (250 μm) containing the SON and surrounding hypothalamus were prepared from mice of either sex (4–6 wk, 17–20 g). The mice were deeply anesthetized with pentobarbital sodium (50 mg/kg ip) and perfused transcardially with a few milliliters of ice-cold, low-Na\(^+\) (NaCl was replaced by an equiosmolar amount of sucrose) artificial cerebrospinal fluid (ACSF) oxygenated with 95% O\(_2\)-5% CO\(_2\). The brain was rapidly removed from the skull, immersed in the ice-cold ACSF for a few minutes, blocked in the coronal plane, and glued to the stage of a vibrating slicer (VT1000s, Leica). The sections were cut into the same sucrose-ACSF slush, transferred to normal ACSF oxygenated continuously at 32–34°C for 1 h, and then maintained at room temperature until recording. The ACSF contained (in mM) 125 NaCl, 2.5 KCl, 2 CaCl\(_2\), 1.0 MgSO\(_4\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), 0.45 ascorbic acid, and 20 g-glucose (pH = 7.4, ~290 mosM). Tests with NiCl\(_2\) or CdCl\(_2\) were performed in phosphate-free ACSF to avoid chelation. The recording chamber was continuously perfused with oxygenated ACSF at ~2 ml/min at 32–34°C.

**Electrophysiological Recordings**

Whole cell patch-clamp recordings were obtained with an Axon Multiclamp 700A amplifier (Molecular Devices) and digitized with a Digidata1322, using pCLAMP 9. Visually directed recordings were made from a modified Olympus BX50WI microscope and a ×40 water immersion lens (4- to 8-MΩ resistance) were prepared from capillary tubing with a horizontal puller (Sutter Instruments). The pipette solution contained (in mM) 140 K-gluconate, 10 KCl, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, 3.5 phosphocreatine, and 0.2 EGTA. The pH of the pipette solution was adjusted to 7.4 with 1 M KOH, and osmolality was adjusted to 285–295 mosM. The intracellular solutions also contained 0.05–0.1% bicytin (Sigma-Aldrich) to further identify the patched cell. Firing patterns were recorded either at rest or by using small current injections to bring the membrane potential near spike threshold. To measure current/potential (I/V) relations, depolarizing pulses were given from a hyperpolarized (~80 mV) membrane potential; hyperpolarizing pulses were given at a potential just below the threshold of the firing. The duration of the pulses was 400 ms. Voltage outputs were filtered at 10 kHz and digitized at 20 kHz. Data were not corrected for a liquid junction potential of ~10 mV.

**Immunocytochemistry and Intracellular Labeling in Slices**

After recording, the slices were fixed for 48–72 h at 4°C with 4% paraformaldehyde and 0.2% picric acid in phosphate-buffered saline (PBS). To identify the boundaries of the SON, either an antibody raised in rabbit against VP-neurophysin (NP) (1:20,000; courtesy of Alan Robinson, UCLA) or a monoclonal OT-NP antibody raised in mouse (PS38, 1:500; courtesy of Harold Gainer, NIH) was used. The secondary antibodies used were Alexa Fluor 568-conjugated goat anti-rabbit IgG (for VP staining) or Alexa Fluor 568-conjugated goat anti-mouse IgG (for OT staining) (Invitrogen, Carlsbad, CA).

Because we observed a large number of small and weakly fluorescent ChAT-eGFP neurons, we compared the distribution of ChAT-eGFP neurons with those immunostained with a ChAT antibody (A144p; Millipore). The A144p antibody was raised in goat, used at a dilution of 1:100, and localized with Alexa Fluor 568 rabbit anti-goat (Invitrogen, 1:200). This antibody has been extensively characterized and used in over 300 published studies.

To identify the bicytin-filled neurons, slices were then incubated overnight at room temperature with avidin-biotin complex (ABC kit, Vector Labs, Burlingame, CA) diluted 1:100 in PBS containing 0.5% Triton X-100. These slices were reacted with a standard diaminobenzidine staining kit (Vector Labs), rinsed, and osmicated for 20 min in 0.05% osmium tetroxide (in PBS) before mounting on the slides with a polyvinyl alcohol (PVA) solution. This procedure yielded a stable reaction product with minimal tissue shrinkage for photomicrography.
and drawing. The images from projections through Z stacks shown in Figs. 5 and 9 were made with a ×20 plan apo objective (0.75 NA) on a Nikon Eclipse 90i microscope with Nikon NIS-Elements software. Filled neurons were reconstructed on a Nikon Optiphot using NeuroLucida (MicroBrightField) and a ×60 water immersion, long-working-distance objective (Olympus plan apo, 1.2 NA).

Confocal Microscopy of GABAergic and Cholinergic eGFP Neurons

Four- to six-week-old GAD65 (n = 3)-, GAD67 (n = 3)- or ChAT (n = 4)-eGFP transgenic mice were anesthetized with pentobarbital sodium (50 mg/kg ip) and perfused transcardially with 4% paraformaldehyde and 0.2% picric acid in PBS. The brain was removed and immersed in the same fixative overnight at 4°C. Coronal or sagittal sections were made with a vibrating slicer (Leica VT 1000s) at 50 μm. Some of the slices were incubated in antibodies to OT-NP, VP-NP, or ChAT (as described above). Slices were mounted in PVA. Fluorescent neurons were imaged with a Zeiss 710 confocal microscope. Positive eGFP neurons were viewed with a laser excitation wavelength of 488 nm, whereas neurons immunostained for OT, VP, or ChAT with a Alexa Fluor 568-labeled secondary antibody were excited with a 561-nm laser line. The images shown in Figs. 1, 6, and 7 were taken with a ×20 objective (NA 0.8). Images of the paraventricular nucleus (PVN) were tiled to encompass left and right sides in the same micrograph. The qualitative assessment of double-labeling of eGFP neurons with the anti-ChAT was made from individual optical sections through image stacks only as far as the antibody visibly penetrated.

Statistics

Comparisons were made with nonparametric statistics (Wilcoxon rank sum test for 2 groups or Kruskal-Wallis nonparametric ANOVA for 3 groups). After three-group comparisons, between-group differences were determined with Steel-Dwass method. Statistics were performed with JMP Pro (SAS Institute). Differences with a P ≤ 0.05 were considered significant. Except for some values given for individual neurons as noted, errors listed are SE.

RESULTS

Distribution of GAD65- and GAD67-eGFP Neurons near SON and PVN

In general, the hypothalamic distribution of GAD65 and GAD67 matched well with that described in studies looking at GAD mRNA with in situ hybridization (Esclapez et al. 1993; Feldblum et al. 1993; Okamura et al. 1990) and immunoreactivity for GAD or GABA (Mugnaini and Oertel 1985). However, we found many more GAD65 than GAD67 neurons in the midanterior regions of the hypothalamus containing the SON and PVN. In particular we found a large number of GAD65-eGFP but very few GAD67-eGFP neurons in the PNZ of the SON (Fig. 1). Our results may reflect the overall intensity difference for mRNA reflected in silver grain counts reported by Feldblum et al. (1993) for many hypothalamic nuclei (greater for GAD65), even though cell numbers in most of these nuclei were similar for GAD65 and GAD67. Although it was previously reported that GAD65 mRNA was found within neurons of the magnocellular component of SON (Feldblum et al. 1993), similar to Theodosis et al. (1986) and Herbison (1994), we typically found GAD65-eGFP only in small neurons near the SON in the PNZ and only rarely inside the SON boundaries (i.e., mixed among large OT and VP neurons). Because of the sparse distribution of GAD67-eGFP neurons in the PNZ, we restricted our recordings to labeled neurons in GAD65-eGFP mice.
Electrophysiological Characteristics of GAD65-eGFP Neurons

We characterized some properties of visualized GAD65-eGFP neurons from whole cell current-clamp recordings in coronal slices. Fluorescent neurons were first briefly observed with 488-nm excitation. It was critical that this exposure was brief, as prolonged viewing resulted in either a failure to patch the cell or in patched cells with the characteristics of damaged neurons (low amplitude, broad spikes, depolarized membrane potential). The effects of prolonged (several minutes) illumination were also visible with DIC in the most extreme cases, with neurons having a flattened, granular appearance.

Membrane properties. We recorded from 32 GAD65-eGFP PNZ neurons and an additional 15 eGFP-negative neurons in the same mice. We measured the input resistance ($R_i$) with a small (+5 mV) voltage step from −70 mV. The average $R_i$ of GAD65-eGFP PNZ neurons was 429.5 ± 61.5 MΩ, and the range was great (122–1,642 MΩ). Small eGFP negative neurons had an $R_i$ of 668.9 ± 137.4 MΩ, which was not different from the positive neurons ($P > 0.304$). To compare with PNZ neurons recorded in rats in a previous study (Armstrong and Stern 1997), we tested neurons with current injection steps at two membrane potentials, one level near spike threshold and another more negative (between −80 and −90 mV). We also passed continuous positive current into neurons that were silent at rest in order to compare their firing pattern with those firing spontaneously. From a hyperpolarized membrane potential, none of the GAD65-eGFP neurons tested exhibited the transient outward rectification in response to depolarizing pulses characteristic of magnocellular SON and PVN neurons (Armstrong and Stern 1997; Bourque 1988; Tasker and Dudek 1991), and all but 2 of 35 tested showed a depolarizing hump that emerged beneath the fast spike threshold, consistent with that observed in some PVN parvocellular neurons (Tasker and Dudek 1991) and PNZ neurons in the rat (Armstrong and Stern 1997) (Fig. 2A). In 8 of 11 eGFP-negative neurons, the LTS was also evoked by this test. As shown in Fig. 2A, the LTS could sometimes reach fast spike threshold, generating multiple fast, large-amplitude spikes.

When neurons were hyperpolarized in current steps from a depolarized membrane potential below spike threshold (between −50 and −60 mV), 19 of 23 GAD65-eGFP neurons tested showed a depolarizing sag indicating an inward rectification (Fig. 2B), with 6 of 7 eGFP-negative neurons tested showing a similar response-characteristic of an $I_h$-type current. The $I/h$ response for neurons without inward rectification was relatively linear. At the offset of the most negative hyperpolarizing pulses, a rebound LTS was also present in all neurons (Fig. 2B). In five of five neurons tested, the LTS and rebound remained after blocking fast Na+ spikes with 0.5 μM TTX (Fig. 3).

In many cases, an LTS similar to what we observed in PNZ neurons is mediated by low-threshold Ca$^{2+}$ currents (see Perez-Reyes 2003 for review). To determine the Ca$^{2+}$ dependence of the LTS in PNZ neurons, we first tested 100 μM NiCl$_2$ ($n = 13$), since 50–100 μM NiCl$_2$ has been shown to block low-threshold Ca$^{2+}$ currents in the SON (Fisher and Bourque 1995; Israel et al. 2008) and in parvocellular PVN neurons (Luther and Tasker 2000). However, 100 μM NiCl$_2$ failed to block or even strongly reduce the LTS in any of these 13 neurons, including 3 neurons tested after TTX (Fig. 3, A–C). The effectiveness of NiCl$_2$ at blocking some currents could be observed, however, by its ability to reduce spontaneous synaptic activity (Fig. 3, D and E). Unfortunately, when we exposed neurons to CdCl$_2$ (200 μM; $n = 7$) to further study the Ca$^{2+}$ dependence of the LTS, the recordings consistently became unstable and we could not complete this assessment.

Firing properties. Firing rate and spike distribution were examined from 1- to 2-min records. Over one-half (21/35) of GAD65-eGFP neurons were spontaneously active, with a mean firing rate of 6.3 ± 0.74 Hz and a coefficient of variation (CV) for interspike intervals (ISI) of 1.07 ± 0.22. These neurons could be loosely grouped into seven neurons that fired in an irregular pattern (CV 0.32–0.77; firing rate 3.3–9.7 Hz), eight bursting neurons, firing bursts of action potentials on an irregular background pattern or phasically with little firing in between (CVs > 1), and six neurons that fired in a more regular, continuous pattern (CV 0.06–0.15; firing rate 5.5–12.9 Hz) (Fig. 4). After continuous depolarizing current injection in another 11 neurons tested, 8 fired irregularly and 3 fired with bursts. The remaining silent neurons were not tested for firing pattern, but all exhibited action potentials upon depolarization. We also recorded from 11 small PNZ neurons that did not express eGFP; of these 9 fired spontaneously, with a mean firing rate of 7.2 ± 1.79 Hz and an ISI CV of 1.19 ± 0.42 (not shown). Most of these nine fired in an irregular pattern ($n = 6$; CV 0.32–0.62; firing rate 4–9 Hz) and a few irregularly with bursts ($n = 3$; CV 1.23–3.33; firing rate 4.4–18.7 Hz), patterns similar to those shown in Fig. 4, B and C. None showed a highly regular pattern. Overall, there were no differences between spontaneously firing eGFP-positive and eGFP-negative neurons with regard to firing rate ($P > 0.7687$) or CV ($P > 0.9223$).

Morphological Characteristics of Filled GAD65-eGFP Neurons

We filled 17 GAD65-eGFP neurons with biocytin. Two examples are shown in Fig. 5. These neurons were similar to those filled earlier with sharp electrodes in hypothalamic ex-
plants (Armstrong and Stern 1997), with fusiform or rounded somata. Most of the neurons had long, thin processes that, in the coronal plane, extended long distances over the SON laterally and medially. While occasionally these processes would pass through the nucleus, we found no apparent terminal processes in the SON. Distinguishing dendrites from axons was often difficult because of the thinness of these processes and also the paucity of visible spines, and many of the identified axons appeared cut near the soma. Thus not only did we not view any processes terminating in the SON, terminal arborizations were not apparent, suggesting that these neurons have projections well beyond the coronal slice. Filled GAD65 neurons exhibited 3.7 ± 0.39 primary dendrites, 7.8 ± 0.9 branches, a dendritic length of 1,606 ± 189 µm, and a somatic area of 185.1 ± 18.6 µm.

**Distribution of ChAT Neurons**

We found two populations of ChAT-eGFP neurons in hypothalamus near the SON. One population had large somata (>20 µm) with multiple dendrites, were brightly eGFP positive, and appeared to be part of the canonical group of basal...
forebrain/diencephalic cholinergic neurons as visualized with ChAT immunohistochemistry (Armstrong et al. 1983; Houser et al. 1983; Woolf et al. 1983). In the PNZ, most of these large neurons lay dorsolateral to the SON, contained in the ventrolateral portion of the lateral and magnocellular preoptic areas and horizontal limb of the diagonal band of Broca (HLDB) rostrally, and caudally in the lateral hypothalamus (LH) and substantia innominata. Some of these large eGFP neurons were found immediately adjacent to magnocellular neurons stained for VP- or OT-NP (Fig. 6). As described originally by Mason and coworkers (Mason et al. 1983; Theodosis and Mason 1988), some of these large ChAT neurons had processes that projected into the SON (Fig. 6B). Large ChAT-eGFP neurons were not observed near the PVN.

A second population of smaller ChAT-eGFP neurons, with a much dimmer fluorescence, was distributed extensively in the rostral hypothalamus, including the PNZ (Fig. 6). These weaker ChAT-eGFP neurons were also observed in the medial part of the PVN, the posterior hypothalamic nucleus immediately caudal to the PVN, and various parts of the mammillary complex. The processes of many of the small ChAT-eGFP neurons were difficult to visualize because of the weaker fluorescence. A very small minority of the smaller ChAT-eGFP-positive somata appeared as bright as the larger neurons. In general, fewer of these smaller neurons were located in the posterior parts of the hypothalamus—the ventromedial nucleus was noticeably devoid of eGFP somata. Other neurons well known to be cholinergic besides those in the basal forebrain, like those of the medial habenula and their axons in fasciculus retroflexus projecting to the interpeduncular nucleus, were strongly eGFP positive.

We incubated hypothalamic slices from two ChAT-eGFP mice with the affinity-purified AB144p polyclonal antibody to determine whether both the brightly and more weakly fluorescent populations of ChAT-eGFP neurons would react for ChAT. Immunofluorescence with this antibody was robust, and

Fig. 5. Filled GAD65-eGFP neurons in the PNZ. A: photomicrograph of biocytin-filled neuron in coronal section taken from a Z stack of nine 1.5-μm steps. The rostral part of the SON and OpC are medial, to left. All processes were relatively thin, and it was not possible to discern an axon from dendrites. The soma lay just lateral to the SON, and a branching process projected over the SON. The reconstruction is shown in C. B and D: another projection from a filled neuron taken from a Z stack of fifteen 1-μm steps (B) and its reconstruction (D). This neuron lay dorsal to the SON, caudal to the neuron shown in A, and the processes projecting ventrolaterally encroached on the SON, whereas a medial process projected over the nucleus. As with the neuron shown in A, it was difficult to distinguish an axon from the other processes. Bars, 100 μm in all frames.

Fig. 6. Confocal image of ChAT-eGFP neurons near the SON in coronal sections of mouse hypothalamus. A: confocal projection from a stack through 1 side of the SON stained for VP-NP to highlight VP neurons (red) (fourteen 0.95-μm optical sections). Numerous large ChAT-eGFP neurons (double arrow, green neurons) are evident and lie close to, but not among, SON neurons. Weakly fluorescent, smaller neurons are also visible (arrow). B: another projection stack through a different section from the same ChAT-eGFP mouse, stained for OT-NP (red) (fourteen 1.05-μm optical sections). Some of the bright, larger ChAT-eGFP neurons (green) lie close to the SON, with processes extending into the dorsal part of the nucleus in the case of the neuron at bottom left. Note in both the presence of more weakly fluorescent green eGFP neurons, some of which are found very close to, even within, the SON (arrows).
the pattern of stained neurons fit that of the canonical ChAT distribution of the basal forebrain mentioned above (as well as the medial habenula) and included many double-labeled neurons. Only eGFP neurons within the same focal plane of anti-ChAT neurons were considered for double labeling, since antibody penetration may not be complete in the middle of the section. Double-labeled neurons in the PNZ lay rostrally in the medial parts of the lateral and magnocellular preoptic areas and the HLD. Caudally the PNZ included the ventromedial aspects of the LH and the substantia innominata, both of which also had a large number of large, bright ChAT-eGFP neurons that were double-labeled for ChAT immunoreactivity. In contrast, the smaller, weakly fluorescent eGFP neurons in the PNZ were not double labeled (Fig. 7). A few of the small neurons were as bright as the large eGFP neurons, but these also did not stain for anti-ChAT.

There were hypothalamic and adjacent regions with smaller eGFP neurons that did double label for anti-ChAT. Posteriorly, there was double labeling in the posterior hypothalamic nucleus, and some parts of the mammillary complex, especially the supramammillary nucleus. A small number of neurons appeared to react for anti-ChAT but did not express eGFP, such as some in the lateral part of the arcuate nucleus. In the adjacent amygdala, a dense, apparently axonal innervation of the basolateral amygdala was visible with both anti-ChAT and ChAT-eGFP double-labeled processes. We found some scattered ChAT-eGFP somata in this region as well, but these neurons did not react with anti-ChAT. In conclusion, the majority of the small ChAT-eGFP neurons in hypothalamus, including those in the PNZ, were not labeled for anti-ChAT.

Electrophysiological Characteristics of ChAT-eGFP Neurons

Membrane properties. We recorded 9 large and brightly fluorescent cells and 12 of the smaller, weakly fluorescent ChAT-eGFP neurons. The larger ChAT-eGFP neurons had an input resistance of $216.4 \pm 47.2$ MΩ, significantly smaller than that of the smaller neurons ($1,406.0 \pm 231.4$ MΩ; $P \leq 0.0002$). The majority (8/9) of the larger neurons did not fire spontaneously, having an average resting potential ($-56.3 \pm 1.9$ mV) that was below spike threshold. In contrast, the majority of the smaller neurons fired spontaneously (see below). All nine of the large ChAT-eGFP neurons were characterized by a transient outward rectification, revealed either with depolarizing pulses when holding the neuron negative, as in Fig. 8A, or at the offset of hyperpolarizing pulses when given from a more depolarized membrane potential, as in Fig. 8C. Seven of these neurons also exhibited a delayed, transient depolarization like that shown in Fig. 8A, but this was much smaller than the LTS observed in parvocellular neurons. In contrast, similar to parvocellular neurons in the GAD65-eGFP mice, 9 of the 11 weakly fluorescent, smaller ChAT-eGFP neurons tested were characterized by a prominent LTS either when depolarized from a hyperpolarized holding potential, as in Fig. 8B, or at the onset of hyperpolarizing pulses from a more depolarized membrane potential, as in Fig. 8D, and showed very little transient outward rectification. About half of the cells of each type exhibited some inward rectification when hyperpolarized to quite negative membrane potentials ($\leq -80$ mV). Another marked difference between the larger ChAT-eGFP neurons and the smaller cells was in the size of the spike hyperpolarizing afterpotential (HAP), which was significantly bigger in the larger neurons ($-20.8 \pm 1.3$ mV) compared with the smaller cells ($-7.7 \pm 0.8$ mV; $P \leq 0.0001$) (Fig. 8).

Firing properties. Eleven of the twelve smaller, weakly fluorescent neurons fired spontaneously, and most (n = 10) exhibited patterns similar to those of the irregular firing parvocellular neurons in the GAD65-eGFP mouse (firing rate = 6.4 ± 0.7 Hz; CV = 0.45 ± 0.06) shown in Fig. 4. One neuron fired regularly (CV = 0.20; firing rate = 9.3 Hz). Bursting neurons were not observed.

Although eight of nine of the larger ChAT-eGFP neurons were silent at rest, they could be depolarized to elicit spike trains. When depolarized to threshold, these nine neurons fired slowly with a mean rate of 2.8 ± 0.2 Hz and a CV of 0.89 ± 0.14. Most (n = 5) of these neurons fired very irregularly (CV 0.55–0.95), but three exhibited bursting patterns (CV 1.06–1.69) and one fired in a highly regular fashion (CV = 0.11). Thus this variability suggests that, like the smaller neurons recorded from the GAD65 mice, firing pattern alone would not distinguish cell types. And although only one large ChAT-

![Fig. 7. Anti-ChAT antibody labels large, but not small, ChAT-eGFP neurons near the SON. A: confocal projection image from a stack through 1 side of the SON (arrow) stained for ChAT (red neurons) (ten 0.86-μm optical sections). Numerous large ChAT-positive neurons are evident, 2 of which are indicated by double arrows. Single arrow points to location of the SON. B: the same section as in A, showing ChAT-eGFP neurons. Note the small, weakly fluorescent eGFP neurons near the SON (arrow) and a smaller but brightly fluorescent eGFP neuron (arrowhead). C: overlap of A and B showing that only large, brighter ChAT-eGFP neurons stain positive with the anti-ChAT antibody. Note that the 1 bright, small eGFP neuron did not stain for anti-ChAT (arrowhead).](http://jn.physiology.org/doi/10.1152/jn.00561.2014)
Dendritic length of 1,363.0 μm primary dendrites with 12.7 branches, characterized by somata that were 324.0 μm in area, 4.4 ± 0.4 primary dendrites with 12.7 ± 1.5 branches, and a total dendritic length of 1,363.0 ± 249.8 μm. Examples are shown in Fig. 9A, B, D, and E. These neurons had large (≥20 μm) polygonal or rounded somata. Most of the dendrites were smooth, often varicose, and not particularly spiny. One or two of the primary dendrites were typically thicker than the other dendrites proximally. Spines were more often found on the distal parts of the dendrite, but in general these neurons would not be characterized as spiny. These neurons were on average located more dorsolateral to the SON, but could be found along its rostrocaudal length. In contrast to the GAD65-eGFP neurons, which had mediolaterally oriented dendrites, large ChAT-eGFP neurons had their dendrites dorsoventrally oriented. The majority of these neurons had extensive axonal arbors that ramified locally (Fig. 9, D and E). However, while dendrites and axons occasionally encroached on the border of the SON, no obvious innervation was noted.

**Morphological Characteristics of Filled ChAT-eGFP Neurons**

Large ChAT-eGFP neurons. The nine large ChAT-eGFP neurons we filled with biocytin and reconstructed were characterized by somata that were 324.0 ± 29.2 μm in area, 4.4 ± 0.4 primary dendrites with 12.7 ± 1.5 branches, and a total dendritic length of 1,363.0 ± 249.8 μm. Examples are shown in Fig. 9A, B, D, and E. These neurons had large (≥20 μm) polygonal or rounded somata. Most of the dendrites were smooth, often varicose, and not particularly spiny. One or two of the primary dendrites were typically thicker than the other dendrites proximally. Spines were more often found on the distal parts of the dendrite, but in general these neurons would not be characterized as spiny. These neurons were on average located more dorsolateral to the SON, but could be found along its rostrocaudal length. In contrast to the GAD65-eGFP neurons, which had mediolaterally oriented dendrites, large ChAT-eGFP neurons had their dendrites dorsoventrally oriented. The majority of these neurons had extensive axonal arbors that ramified locally (Fig. 9, D and E). However, while dendrites and axons occasionally encroached on the border of the SON, no obvious innervation was noted.

Small ChAT-eGFP neurons. In contrast, the eight filled smaller ChAT-eGFP neurons (soma area = 117.6 ± 13.7 μm) exhibited only 2.5 ± 0.5 primary dendrites, with 4.0 ± 1.2 branches, and a total length of 531.7 ± 215.2 μm. The somata of these neurons were rounded or fusiform in shape. Three examples are shown in Fig. 9C. Although soma size was similar to GAD65-eGFP neurons, the dendritic trees of these neurons were much less extensive, having fewer primary dendrites, fewer branches, and only about one-third of the total dendritic length, suggestive of a different morphological phenotype. The dendrites of the smaller neurons were seldom spiny and often varicose. Thus, although similar in some electrophysiological properties to GAD65-eGFP neurons (like the LTS), these two neuron types had morphologies very different from one another.

**Morphological Comparisons Across Cell Types**

A comparison of the two ChAT-eGFP groups with the filled GAD65-eGFP neurons revealed several differences. Not surprisingly, the three groups differed in soma area (P ≤ 0.0002), with both parvocellular neurons significantly smaller than large ChAT-eGFP neurons (P = 0.0018 for small ChAT neurons, P = 0.0035 for GAD65-eGFP neurons). There was a difference in the number of primary dendrites (P ≤ 0.0104) that was only significant between the small and large ChAT-eGFP neurons.
neurons \( (P = 0.025) \). The number of branches differed \( (P \leq 0.0014) \), with between-group comparisons showing that large ChAT-eGFP neurons had the most branches compared with either the small ChAT-eGFP neurons \( (P = 0.0078) \) or the GAD65-eGFP neurons \( (P = 0.0396) \). Interestingly, GAD65-eGFP neurons had more branches than small ChAT-eGFP neurons \( (P = 0.0467) \). Total dendritic length also varied significantly across groups \( (P < 0.0072) \). However, unlike branching, there was no difference in dendritic length between GAD65-eGFP and the large ChAT-eGFP neurons, whereas small ChAT-eGFP neurons had much smaller dendritic trees than either GAD65-eGFP \( (P = 0.013) \) or large ChAT-eGFP \( (P = 0.0483) \) neurons.

**DISCUSSION**

With the advantage of transgenic mice labeled for the synthetic enzymes for GABA and acetylcholine, we have been able to extend our original observations of unidentified PNZ neurons by comparing the morphology and electrophysiology of these two classes of neurons near the rodent SON. Our findings suggest these broad results: 1) the three groups of parvocellular neurons we examined in this region overwhelmingly possess an LTS but otherwise differ in morphology; 2) large ChAT neurons exhibit characteristics very different from the smaller PNZ neurons, characteristics similar to canonical basal forebrain ChAT neurons; and 3) as in our previous investigation from unidentified neurons in the rat PNZ (Armstrong and Stern 1997), we were unable to demonstrate direct axonal terminal-type innervation from this region to the SON; however, reconstructions were done from slices and processes were undoubtedly severed. In contrast, as discussed below, processes, probably both axons and dendrites, did encroach on the SON.

**Expression of GAD65-eGFP and GAD67-eGFP in PNZ**

The expression of GAD65-eGFP neurons, which we found much more plentiful than GAD67 in hypothalamus and adjacent structures, reasonably matched previous studies using in situ hybridization in rats (Feldblum et al. 1993). This included a large number of neurons in the anterior nucleus and medial hypothalamus, medial preoptic area, suprachiasmatic nucleus, zona incerta, and bed nucleus of the stria terminalis (also see Okamura et al. 1990; Roland and Sawchenko 1993). Within the PNZ, scattered GAD-positive or GABA-positive neurons also have been observed immunohistochemically (Herbison 1994; Iijima et al. 1986; Theodosis et al. 1986). While not densely distributed, the GAD65 neurons nevertheless were plentiful and easily located in the PNZ in all slices, where they lay more dorsolaterally than dorsomedially to the SON, at the ventral aspect of the LH. Some neurons encroached on the dorsal aspect of the SON, but these were very sparse and typically not as brightly fluorescent as those clearly more dorsal in the PNZ. Some of these may correspond to putative GABA neurons containing GABA transaminase described by Iijima and Kojima (1985) in the SON.

**Electrical Properties of GAD65-eGFP Neurons**

The line of transgenic mice we used has been valuable in characterizing developmental, morphological, and electrophysiological attributes of GABAergic neurons in many areas of brain and spinal cord since its creation in 2004 (López-Bendito et al. 2004), and single-cell polymerase chain reaction has verified GAD67 and/or GAD65 mRNA in many eGFP-positive neurons in the hypothalamus and zona incerta (Shin et al. 2007). GABAergic neurons in the LH, which may include the
PNZ, have been extensively characterized both morphologically and electrophysiologically in these mice (Karnani et al. 2013). One type of LH GABAergic neuron had an LTS with a profile similar to the great majority of PNZ GABAergic neurons. In the anterior hypothalamic nucleus, the ventrolateral aspects of which could also include the PNZ, some GABAergic neurons were found to have an LTS and exhibit short bursts. However, these neurons were studied in adrenalectomized animals with exposure to corticosterone, where the LTS was more prominent after mineralocorticoid receptor activation (Shin et al. 2011).

In some regions of the brain, LTS profiles similar to those we observed are blocked or strongly suppressed by low (50–100 μM) concentrations of Ni2+, compatible with the expression of at least one class of T-type Ca2+ channels (CaV3.2) (Perez-Reyes 2003). In hypothalamus, this appears the case for the LTS and the T current found in one class of parvocellular PVN neurons (Luther and Tasker 2000) and in magnocellular neurons in the rat SON (Fisher and Bourque 1995; Israel et al. 2008). However, in other cases, the LTS or T current may be sensitive only to higher concentrations of Ni2+ (Perez-Reyes 2003). While the LTS in the PNZ GAD65-eGFP neurons remained after TTX, it was not blocked by 100 μM NiCl2, suggesting an underlying channel type different from CaV3.2. While this could certainly result from a different subtype of Ca3 channel, it is also noteworthy that Han et al. (2005) found fast, TTX-resistant Na+ currents in GABAergic neurons of the basal forebrain associated with the expression of Nav1.5 subunits, and such channels could contribute to an LTS.

As for the profile of the LTS itself, there seemed little difference between the GAD65-eGFP positive and -negative neurons, or from what we observed previously in unidentified neurons with sharp electrodes (Armstrong and Stern 1997). With regard to firing pattern, a range was observed, with an emphasis on irregularly firing and some bursting neurons. However, a regular, oscillatory bursting pattern characteristic of some neurons with an LTS, such as those in thalamus (e.g., Huguenard and Prince 1992; Kim and McCormick 1998), was not observed. However, this pattern is likely highly voltage dependent and possibly network driven; thus its absence in our recordings must be viewed with caution.

**Morphology of GAD65-eGFP Neurons**

While the morphology of many GAD65-eGFP neurons resembled some of the unidentified PNZ neurons we previously reconstructed from hypothalamic explants (Armstrong and Stern 1997), the use of slices restricted visualization of extended processes compared with the explan. Notable, however, was that most of the neurons we filled in the present study were aspiny, compared with the ~50% of neurons in the explan that were densely spiny. While many of the neurons had extensive processes projecting mediolaterally, similar to a majority of the neurons in the first study, the same degree of axonal or dendritic branching was not apparent, again most likely because of the restricted dimension of the slice vs. explants, although we cannot rule out a difference between rats and mice in this regard. In neither study could we verify a local, terminal projection to the SON, even though processes passed near or even with SON boundaries in both cases. Since processes in passage, either dendritic or axonal, may take up extracellular tracers, it remains unsettled whether PNZ neurons are a major source of the extensive GABAergic innervation of the SON, or whether GABAergic afferents pass through here (and thus could be activated from electrical stimulation in slices, for example) on their way to the SON. At least some GABAergic PNZ neurons are likely to innervate the SON, since local glutamate stimulation can produce inhibitory synaptic currents in a small minority of neurons tested (Wuarin 1997). It has been demonstrated, however, that PNZ neurons can also project to other regions, such as the LH (Gritti et al. 1994). Interestingly, its close proximity to, and the presence of processes very near and sometimes within, the SON suggest that PNZ neurons could be targets of dendriclly released VP and OT (Ludwig and Leng 2006), which could then contribute to some effects of magnocellular neuron activation not otherwise explained by the well-known neurohypophysial projection (Neumann 2007).

**Expression of ChAT-eGFP in PNZ**

The brightest, larger ChAT-eGFP neurons near the SON appeared part of the canonical, basal forebrain cholinergic system, the most ventral and caudal aspects of which included the PNZ in the LH (Armstrong et al. 1983; Houser et al. 1983; Woolf et al. 1983). These large neurons were overwhelmingly positive for anti-ChAT. In contrast, the numerous smaller ChAT-eGFP neurons in the PNZ, the great majority of which were dimly fluorescent, were not immunoreactive. Some immunohistochemical studies have reported a relatively broad distribution of ChAT-positive neurons in rat hypothalamus, including many smaller neurons (Rao et al. 1987; Rodriguez-Sierra and Morley 1985; Tago et al. 1987). The presence of the smaller, ChAT-eGFP PNZ neurons could be the result of transgenic “leakiness” (see Challen and Goodell 2008 for a discussion of this issue), although to our knowledge this has not been reported for this transgenic strain. Alternatively, ChAT-eGFP expression may allow greater sensitivity for low ChAT levels. Thus, at present, we cannot corroborate whether the small PNZ ChAT-eGFP neurons we observed were truly cholinergic, nor can we exclude this possibility. What is clear, however, is the marked difference in the electrophysiological properties of the large neurons that were double labeled and these smaller neurons that were only eGFP positive.

**Electrical Properties of ChAT-eGFP Neurons**

The larger ChAT-eGFP neurons we recorded differed from the parvocellular PNZ types, including GAD65-eGFP neurons, by exhibiting 1) a prominent transient outward rectification that delayed spiking when neurons were depolarized from a relatively hyperpolarized membrane potential; 2) a large spike HAP; and 3) a weak transient depolarizing potential, smaller than the LTS recorded in parvocellular neurons. In general, our results are in good agreement with those of Hedrick and Waters (2010), who recorded from immunohistochemically identified ChAT neurons in mice, and Unal et al. (2012), who recorded from ChAT-eGFP neurons in the same transgenic strain as in this study. Others have reported strong transient outward rectification in ChAT neurons in septum and nucleus basalis in rats and guinea pigs (Khateb et al. 1993; Markram and Segal 1990; Matthews 1999; Tkatch et al. 2000; Unal et al. 2012) as well as a large spike afterhyperpolarization (AHP) (Griffith and
Matthews 1986; Matthews 1999; Unal et al. 2012). However, in many studies, forebrain ChAT neurons have also been found to exhibit a prominent LTS and/or a high density of T channels (Alonso et al. 1996; Gorelova and Reiner 1996; Han et al. 2005; Khateb et al. 1992; Unal et al. 2012). This difference across studies may lie in the recent discovery by Unal et al. (2012) that ChAT basal forebrain neurons may be classed into two types: a late-spiking type that exhibits prominent transient outward rectification (delaying the first spike on a depolarizing step) and a large spike AHP sensitive to the SK channel blocker apamin and an early-spiking type that exhibits much more T-type calcium channel current and a smaller spike AHP compared with late-spiking neurons. Clearly most large ChAT neurons we recorded in the PNZ resembled late-spiking neurons. Tkatch et al. (2000) have shown that the amount of A-type K+ current (I_A) in large basal forebrain ChAT neurons is directly correlated with the amount of the K_v4.2 subunit expressed, which likely underlies the transient outward rectification. Although Unal et al. (2012) found that early- and late-spiking types have similar amounts of I_A, late-spiking neurons showed a strong relationship between the time-dependent inactivation of I_A and the time delay for spiking. Furthermore, blockade of the T-channel activity produced the late-spiking profile in early-spiking neurons, suggesting the temporal overlap of these transient Ca^{2+} and K+ currents during the initial depolarization in early-spiking neurons.

The great majority of the more dimly fluorescent, smaller ChAT-eGFP neurons exhibited little transient outward rectification and a prominent LTS—very similar to the GAD65-eGFP-positive and -negative parvocellular neurons. The transient, delayed depolarization we observed in some large ChAT-eGFP-positive neurons did not produce bursts of spikes. Since the expression of an LTS would likely be competitive with the strong transient outward rectification (Unal et al. 2012), it is possible that large mouse ChAT neurons have the underlying T-type or related inward currents superimposed on a strong I_A-type current. The small ChAT-eGFP neurons exhibited electrical properties largely indistinguishable from GAD65-eGFP neurons, or the eGFP-negative neurons we studied in those animals.

Morphology of ChAT-eGFP Neurons

The large ChAT neurons appear to be an extension of the basal forebrain population long known to project to the cortex and other forebrain regions (e.g., McKinney et al. 1983). Morphologically, we found these neurons resembled those from the Golgi study of Dinopoulos et al. (1988), who described ~50% of basal forebrain neurons as having large polygonal or triangular somata with three to five dendrites possessing several branches—this description also matches that of identified cholinergic neurons in the same general region of guinea pig basal forebrain by Alonso et al. (1996) and Manns et al. (2000). Like previous studies, we found the dendrites of most neurons to be relatively smooth, often varicose, and only occasionally studded with spines. Dinopoulos et al. (1988) also found neurons in nucleus basalis with smaller rounded or fusiform somata that had much less extensive dendritic trees, reminiscent of the smaller neurons we observed for the smaller ChAT-eGFP neurons. However, the noncholinergic neurons studied by Alonso et al. (1996) in substantia innominata were not very different in size or in dendritic morphology from their ChAT-positive neurons but were found generally ventral to the latter. The smaller ChAT neurons from which we recorded had morphologies different from both the GAD-65 neurons and the larger ChAT neurons, with a much more restricted dendritic tree, both in the numbers of primary dendrites as well as the extension of these dendrites, than either of these other cell types. This suggests that despite electrophysiological similarities these represent two distinct groups of parvocellular neurons. As with GAD65-eGFP neurons, we found no evidence for terminal-type axonal projections to the SON for either type of ChAT neuron, but again, processes (dendrites or axons) could sometimes be observed encroaching on the SON.

Firing Properties of PNZ Neurons

In general, the spontaneous firing patterns of the parvocellular neurons we recorded would not be useful for distinguishing the various cell types. A great diversity was found in the GAD65-eGFP neurons, 40% of these being silent, and of the spontaneously firing neurons, patterns ranged from regular to irregular and of the latter included many bursting neurons. Although regular bursting activity has previously been associated with an LTS in cholinergic neurons in guinea pig (Khateb et al. 1992) and broadly in thalamic neurons (see Contreras 2006 for review), we found no such activity. However, repetitive bursting in other LTS neurons is often the result of network activity (Kim and McCormick 1998) and, even when not, is voltage dependent (Beatty et al. 2012).

The largest difference we noted among the various cell types was that only one of nine large ChAT neurons fired spontaneously, compared with GAD65-eGFP neurons, small eGFP-negative neurons, and small ChAT-eGFP neurons, where spontaneous activity was present in 60–90% of the cells. Even when prompted to fire, the larger ChAT-eGFP neurons fired more slowly than the smaller PNZ cell types but could exhibit patterns of activity (regular, irregular, bursting) that would be indistinguishable from the smaller cell types, including those recorded in the GAD65-eGFP mice. The low amount of spontaneous activity we observed in the larger ChAT neurons is consistent with a previous study in mice (Hedrick and Walters 2010).

A Cautionary Note for Dissociated SON Preparations

Regarding Cholinergic Neurons

Magnocellular SON neurons (Bourque 1988), especially VP cells (Fisher et al. 1998; Stern and Armstrong 1996), exhibit a strong transient outward rectification that distinguishes them from nearby parvocellular neurons. A similar distinction has been noted between the magnocellular neurosecretory and other neurons in the PVN, where neurosecretory parvocellular neurons, while exhibiting little transient outward rectification, do not exhibit the LTS characteristic of preautonomic PVN neurons (Luther et al. 2002; Stern 2001). As elsewhere in the brain, dissociated cell preparations of the SON have proved valuable for the initial characterizations of a variety of ion channel currents because of favorable space clamp. In general, these neurons were chosen on the basis of their large size, which correlated with larger size of identified magnocellular OT or VP neurons; these large neurons were characterized by...
prominent transient outward rectification (Oliet and Bourque 1992). Fisher and Bourque (1998) later characterized the underlying $I_o$ in identified OT and VP neurons. In basolateral cholinergic neurons, $I_o$ is correlated with mRNA abundance for the K$_{4,2}$ channel type, the subunit also suggested for $I_o$-type current in magnocellular SON neurons based on immunocytochemistry (Alonso and Widmer 1997). The close proximity of some of the ChAT neurons to the SON, their similarly large somata, and their prominent transient outward rectification mean that without immunochemical or some supplementary verification of their neurosecretory phenotype, they could be mistaken for OT or VP neurons. Although on average SON neurons have fewer (2 or 3) primary dendrites than the ChAT neurons (4 or 5 dendrites), there are some magnocellular neurons (4 or 5 dendrites), there are some magnocellular neurons with as many as four or five primary dendrites (Armstrong 1995; Randle et al. 1986; Smith and Armstrong 1990; Stern and Armstrong 1998), and three of the nine ChAT neurons we filled had only three primary dendrites. Finally, the dissociation procedure typically ensures that the original dendritic morphology will not be represented in the acutely isolated neuron.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.W. and W.E.A. conception and design of research; L.W. and W.E.A. performed experiments; L.W. and W.E.A. analyzed data; L.W., M.E., G.S., and W.E.A. interpreted results of experiments; L.W. and M.E. drafted manuscript; L.W., M.E., G.S., and W.E.A. edited and revised manuscript; L.W., M.E., G.S., and W.E.A. approved final version of manuscript.

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